Hyaluronidase production in *Streptococcus milleri* in relation to infection

P F UNSWORTH

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SUMMARY   One hundred and seven (41%) of 262 isolates of *Streptococcus milleri*, from human sources, produced hyaluronidase. Hyaluronidase production was commoner in β haemolytic isolates 32 of 39 (82%), many of which were of Lancefield group F. But hyaluronidase was also found in α and non-haemolytic isolates, and in groups A, C, G, and non-groupable isolates. There was a strong association between hyaluronidase production and isolation from known internal abscesses (48/58, 83%) compared with isolates from the normal flora of uninfected sites (24/97, 25%). Isolates from 15 patients with endocarditis were uniformly negative, although 13 of 25 (52%) isolates from dental plaque produced the enzyme.

Production of hyaluronidase may therefore be an important determinant in the pathogenicity of infection by *S milleri* and could be helpful in predicting the likelihood of deep purulent lesions in isolates from blood culture.

The species name *Streptococcus milleri* was coined by Guthof.[1] Colman and Williams argued for inclusion in the taxon of strains of differing Lancefield antigen— that is, whether they had Lancefield group antigen A, or C, or F, or G, or none; and whether they were α−, β−, or non-haemolytic.[2] Parker and Ball showed that when the taxonomic criteria of Colman and Williams were applied to streptococci (and a few aerococci) from deep lesions in man, then *S milleri* was strongly associated with clinically purulent lesions.[3] In further studies of a selection of some 18 strains thus classified as *S milleri* (Streptococcus Reference Laboratory, Colindale, London) and of varying Lancefield and haemolytic states, it was found that all strains lacked streptolysins O and S and anti-DNase B, but in a few cases possessed hyaluronidase. A possible correlation was noted between hyaluronidase production and stated presence of pus in the patient from which the strain was isolated (Unsworth, unpublished data). Therefore it was decided to study hyaluronidase production in a large number of isolates of *S milleri* and to examine the relation of this enzyme to clinical data on the strains.

Material and methods

One hundred and sixty five isolates from infected patients and routine clinical laboratory samples were

collected by Dr MT Parker, Streptococcus Reference Laboratory, Colindale, London, from isolates submitted from various hospitals and Public Health Laboratory Service laboratories in Britain, and a few isolates from overseas. There were also 13 isolates from normal faeces[4] and 13 from dental plaque isolated by Dr JM Hardie.

The 191 isolates, including faecal and dental plaque isolates, were stored at −20°C in blood broth containing 16% glycerol. Some strains were subcultured repeatedly from glycerol blood broth after storage at −20°C for periods of various months, and then reused for hyaluronidase production. Between 1974 and 1976 61 isolates were identified, as described by Parker and Ball,[3] and subsequently another 130, as described by Waitkins, Ball and Fraser.[5] After May 1979 32 strains were not tested for Lancefield Group antigen. Virtually every strain hydrolysed arginine and gave a positive Voges Proskauer reaction. Most fermented aesculin and lactose but not raffinose, mannitol, or sorbitol. Most had no Lancefield group antigen. Just over half tolerated 10% bile, were non-haemolytic, and were unaffected in their growth by carbon dioxide. (Effect of carbon dioxide was not recorded for 34, including 13 plaque isolates.) The frequency of various features is shown in table 1.

Seventy one strains from normal flora were isolated at Tameside General Hospital in 1986 and 1987. Swabs were incubated overnight at 37°C in 25 g/l Nutrient Broth No 2 (Oxoid Ltd) with added yeast

Table 1 *Cultural, serological, and biochemical characters of 191 isolates of S milleri*

Character	% Frequency of stated character
α haemolytic	26
β haemolytic	20
Non-haemolytic	54
Lancefield Group A	3
Lancefield Group C	3
Lancefield Group F	20
Lancefield Group G	4
Lancefield Group None	70
Carbon dioxide requirement	25
Carbon dioxide enhancement	15
Carbon dioxide not enhancing	60
Salt tolerance: 6·5%	2
Bile tolerance: 40%	40
Bile tolerance: 10%	57
Arginine hydrolysis	98
Voges-Proskauer reaction	99
Acid from:	
Sorbitol	1
Mannitol	10
Lactose	70
Raffinose	20
Aesculin	76

extract 0·3% and Streptococcus Selective Supplement (Oxoid), giving a final concentration of colistin sulphate 10 mg/l and oxolinic acid 5 mg/l, crystal violet 1/500 000 final concentration, and horse blood 5%. Subcultures of broths were incubated in air and carbon dioxide 5% at 37°C overnight on Columbia blood agar (Oxoid). Isolates were identified as *S milleri* by API 20 Strep kits according to the manufacturer's instructions (API Laboratory Products).

Table 3 categorises the clinical importance of isolates into four groups. Fifteen were from blood cultures of patients with endocarditis. Fifty eight were from abscesses or frankly purulent lesions, directly sampled or isolated from blood cultures. Abscesses occurred in the liver (n = 15), brain (n = 9), subdural space (n = 1), lung (n = 3), pleural empyemata (n = 2), parapharyngeal space (n = 1) and psoas sheath (n = 1). There were two isolates from patients with meningitis. Gastrointestinal abscesses comprised eight from the appendix or appendectomy wound and one each from the left iliac fossa, pelvis, pancreas and from the perianal, ischiorectal and subhepatic areas. Two gastrointestinal isolates were from pus in the cystic duct and from the blood culture of a patient with pancreatitis. Two others, both from blood cultures, were from a patient with fever, sweating, and hypotension following resection of a rectal carcinoma; and from a patient with generalised peritonitis due to a perforated gastric ulcer. There were six isolates from cutaneous abscesses: two paronychias, a preauricular abscess, a breast abscess and two injection-site abscesses—one in a diabetic the other in a drug addict.

Miscellaneous clinical samples comprised eight from blood cultures of febrile patients; 30 from appendices or appendectomy wounds not known to have been frankly purulent; eight single isolates from the pleural cavity, a prosthetic hip joint, a chronic submaxillary abscess, a human bite wound, a post-gastrectomy wound, a post-thoracotomy chest drain, an abscess below an "old" abdominal wound and an unspecified wound; 17 urinary strains and one from blood culture of a patient with urinary infection; 10 vaginal isolates, seven from women with vaginal discharge, two of whom had intrauterine contraceptive devices, and one from a patient with suspected salpingitis; nine isolates from throat swabs; one from sputum; seven from blood cultures of subjects within minutes of dental extraction; and one rectal isolate.

Isolates from normal flora were mostly from the study at Tameside General Hospital of patients without clinical infection who had not taken antibiotics in the preceding six weeks. These comprised isolates from the vagina (n = 27), dental plaque (n = 12), gingival crevice (n = 9), rectum (n = 11), anus (n = 2), sigmoid colon (n = 3), sigmoid diverticulum (n = 2), gall bladder (n = 1) and from the throat (n = 4). Those from the sigmoid colon, sigmoid diverticulum, and gall bladder were obtained at necropsy. The isolates from dental plaque and gingival crevice were mostly from 11–15 year olds, from the lower incisors. Added to these were 13 dental plaque isolates kindly supplied by Dr JM Hardie, London Hospital Dental College. These were from the distal surfaces of the upper first premolars of 11–14 year old school children and each strain came from a different subject. Also included were 13 isolates from the faeces of healthy adults.[4]

Strains were grown on blood agar and checked for purity, then inoculated into 5 ml or 50 ml bottles of broth and incubated overnight (or for 48 hours if visible heavy growth was required) at 37°C in air with added carbon dioxide 5% v/v. The broth was Difco Todd Hewitt Broth with added Difco Neopeptone 2% w/v and buffering $Na_2HPO_4.2H_2O$ 0·74 g/l and NaH_2PO_4 $2H_2O$ 0·13 g/l; additives prevented proteinase production.[6] The pH of the broth was adjusted to 7·4 before autoclaving for five minutes at 115°C and sterility was checked by incubating at 37°C in air for 18 hours. Samples of some batches of broth had been tested for ability to inhibit strains of *S pyogenes* from producing proteinase.[7] Broth cultures of *S milleri* were centrifuged until the supernatant was clear, which was then stored at 4°C with merthiolate (final concentration 1/5000). The effect of adding merthiolate to hyaluronidase titre and of storage time at 4°C and 37°C was checked by retesting the titres of certain supernatants.

Broth supernatant (0·025 ml) was added to the first and second of a row of wells of a microtitre plate and

double diluted from the second well with cold distilled water. A further volume of 0·025 ml of cold distilled water was added to each well, followed by 0·05 ml of potassium hyaluronate (Bacto-AHT substrate, Difco Ltd, Chertsey, Surrey) prepared as follows: 8 ml of cold (4%) diluted India ink (20 ml distilled water to 0·01 ml of India ink) added to the Bacto-AHT phial of freeze-dried substrate according to the manufacturer's instructions except for additions of India ink[8]. The microtitre tray was shaken on a microtitre shaker to mix the solutions, then incubated at 37°C for 20 minutes. The tray was then cooled at 4°C for 30 minutes and 0·025 ml of cold (4°C) normal acetic acid added to each well. The trays were again shaken to mix the solutions. Presence of a black clot indicated intact hyaluronate substrate, which was clotted by addition of acid. Absence of clot showed presence of hyaluronidase in the well, and the titre of hyaluronidase was the highest dilution failing to show any clot. Four known isolates of *S milleri* were used as positive controls.

Results

PRODUCTION AND STABILITY OF HYALURONIDASE IN SUPERNATANTS OF *S milleri* BROTH CULTURES

Of the total 262 isolates of *S milleri* tested, 107 (40·8%) produced hyaluronidase. Of 29 strains tested initially, hyaluronidase was produced in broth supernatant by 13 (45%) of the strains, in titres ranging from 4 to 128 (mostly 16 to 32). The titre of hyaluronidase remained stable for up to 14 weeks with no or little change, whether stored at 4°C or 37°C for each of five strains tested. Hyaluronidase titres were unaffected by adding merthiolate. A few strains which were repeatedly recovered from glycerol blood broth after some months of further −20°C storage were unimpaired in their ability to yield hyaluronidase when recultured in broth at 37°C.

ASSOCIATION BETWEEN HYALURONIDASE PRODUCTION AND HAEMOLYTIC REACTION AND LANCEFIELD GROUP ANTIGEN STATE OF THE STRAINS

This is shown in table 2. Production was most common (32 of 39, 82%) in β haemolytic isolates, many of which were group F. Hyaluronidase was also found in 33–60% of α- and non-haemolytic and in group A, C, G and non-groupable isolates.

FREQUENCY OF HYALURONIDASE PRODUCTION IN RELATION TO SOURCES OF ISOLATES AND ASSOCIATED DISEASES

Isolates from internal abscesses and frankly purulent lesions frequently produced hyaluronidase (table 3). This was most noticeable in isolates from deep abscesses: 15 of 15 (100%) from liver abscesses; nine of 10 (90%) from brain and subdural abscesses; seven of eight (87%) from abscess of the appendix or appendectomy wound; eight of 10 (80%) from the other gastrointestinally associated purulent diseases; five of seven (71%) from miscellaneous deep collections of pus in the lung, pleural space, parapharyngeal space and psoas sheath; and three of six (50%) cutaneous abscesses.

In sharp contrast, strains from normal floral sites produced hyaluronidase much less often (table 3). The average was 25%, but the figure was much lower in faecal (one of 13, 8%) and vaginal samples (one of 27, 4%). It was somewhat higher in the rectum with three (27%) of 11 isolates being positive. Dental plaque isolates were an exception, and the frequency differed in strains from Dr Hardie's study, of which 12 of 13 (92%) were positive, and the Tameside Hospital study of which one of 12 (8%) was positive; two of nine (22%) gingival crevice isolates also produced

Table 2 *Association between hyaluronidase production of S milleri, haemolysis, and Lancefield antigen in 191 isolates*

| Haemolysis | Lancefield group | | | | | | |
	A	C	F	G	None	Not tested	Total
α-	0/0	0/0	0/ 3	1/1	11/17	10/ 6	22/27
β-	1/0	3/0	17/ 0	0/3	8/ 4	3/ 0	32/ 7
Non-haemolytic	0/3	0/2	4/ 8	0/2	37/24	1/12	42/61
Total	1/3	3/2	21/11	1/6	56/55	14/18	96/95

Ratio = hyaluronidase positive:hyaluronidase negative isolates, as absolute numbers of isolates.

Table 3 *Sources and probable clinical importance of isolates of S milleri and production of hyaluronidase*

| Categories of isolates ranked in order of probability of their being associated with infection | No of isolates from | | Total No of isolates | No (%) of isolates hyaluronidase positive | |
	Blood cultures	Direct samples			
Endocarditis	15	0	15	0	
Known abscesses and frankly purulent lesions	9	49	58	48	(83)
Miscellaneous clinical samples	16	76	92	35	(38)
Normal flora	0	97	97	24	(25)
All the above	40	222	262	107	(41)

hyaluronidase.

Isolates from miscellaneous clinical samples gave intermediate results (table 3). Isolates from appendices or appendectomy wounds, which were not stated to have been frankly purulent, produced hyaluronidase in 14 of 30 (47%). From the throat and sputum five of 10 were positive for hyaluronidase. Four of seven isolates from blood cultures collected after dental extraction produced hyaluronidase. Only 10% of vaginal and 11% of urinary isolates were positive. Strikingly, isolates from patients with endocarditis were all hyaluronidase negative (table 3).

Comparison of the isolates from known abscesses and frankly purulent lesions with all the others showed that far more of the former were hyaluronidase producers: 83% *v* 29%, $\chi^2 = 54\cdot2$, p = < 0·001. When the abscess and frank pus isolates were compared with those from normal flora, the frequencies were 83% *v* 25%, $\chi^2 = 40\cdot6$, p = < 0·001.

Of the blood culture isolates, four of seven dental bacteraemia isolates produced hyaluronidase. Nine blood culture isolates were deemed to be from deep purulent infections. The one from a case of meningitis was a non-producer. All the remaining eight (four liver and one lung abscess and three gastrointestinal tract related pus) produced hyaluronidase.

Discussion

S milleri from abscesses produced hyaluronidase with a frequency of 50% to 100% (mean 83%), the higher the frequency, the deeper the abscess site—all 15 isolates from liver and 90% of brain and subdural abscesses. In contrast, *S milleri* from sites of normal flora produced hyaluronidase with a frequency of 4% to 52% (mean 24%). Low frequencies of 4% and 8% were found in vaginal and faecal isolates; 28% in large bowel and anal samples; 22% in gingival crevice samples, and 8% to 92% (mean 52%) in dental plaque samples. The variation in the rate of isolation in dental plaque samples may reflect different dental sampling sites.

The strong association between hyaluronidase production and abscesses containing *S milleri* suggests that hyaluronidase has an important role in their formation. *S milleri* hyaluronidase probably dissolves the intercellular cement or connective tissue matrix, of which hyaluronic acid is an important component.[9] Clearly, the formation of an abscess requires the destruction of body tissue.

Abscesses in which *S milleri* is found may be multiple and large. These abscesses are often indicative of mixed infection, *S milleri* commonly being associated with anaerobes—in liver and brain abscesses.[10-13] Alderson *et al* point out that *Bacteroides melaninogenicus* also produces hyaluronidase.[13]

Perhaps hyaluronidase in mixed infections may be produced by one or other of the organisms to fulfil the enzyme's role in pathogenesis.

In dental root abscesses there is evidence to suggest that *S milleri* may be important in initiating the infection and establishing conditions for subsequent flourishing of anaerobes.[14] In Meleney's synergistic gangrene hyaluronidase is also relevant, and streptococci which are probably *S milleri* (sometimes mistakenly called microaerophilic when in fact capnophilic[15]) play a part.[16]

Endocarditis is a disease in which abscess formation is only rarely present.[17] This rarity is consistent with the absence of hyaluronidase production in the 15 isolates studied here, and with the low frequency of *S milleri* among all streptococcal causes of endocarditis.[3] Perhaps absence of hyaluronidase in these isolates may correlate with some other factor favouring endocarditis.

Blood cultures are of value in identifying patients with deep abscesses due to *S milleri*—for example, those in the liver—which can be of insidious onset and often missed until necropsy.[18] Isolates from blood cultures which produce hyaluronidase and which are not collected soon after dental extraction, or similar, related dental manoeuvres, would seem to be highly likely to originate from deep purulent lesions. Thus testing for hyaluronidase production in clinical blood culture isolates will help suggest the presence of collections of pus.

Presumably the hyaluronidase production in isolates from genuine infections may be a result, either of producers having that preferential advantage which enables them to cause the infection or, in some way, of infected tissues stimulating the production of the enzyme. In either case hyaluronidase production in such isolates is a marker of genuine infection.

It seems that strains of *S milleri* may reach sites of deep infection by direct entry from adjacent surface carriage sites, or indirectly via the blood, lymphatics, or along tissue planes. Direct entry, particularly from the alimentary tract, is referred to by Admon *et al*,[19] who also refer to local overgrowth of *S milleri* in lesions such as perforations of the gut when antibiotics may select out this organism. Other studies have reported isolation of *S milleri* from a variety of superficial body sites.[20 21] The present study adds data on certain sites in persons without either clinical infection or a recent history of antibiotic ingestion. One site which seems likely to be an important source of bacteraemic spread of *S milleri* is dental plaque and associated peridental tissues. Plaque has a high prevalence of hyaluronidase positive isolates. *S milleri* is found in the blood following dental extraction[22] and some such strains produce hyaluronidase. Both dental plaque and brain and liver abscesses frequently con-

tain *S milleri* and anaerobes. The species of anaerobe, such as *Bacteroides melaninogenicus/oralis* and *Fusobacteria* present in some liver[12] and some brain[13] abscesses are more consistent with a possible gingival origin than one in the bowel.[23–25] Mello and Raff found evidence of liver abscess in two of three patients with brain abscess,[26] which would favour blood borne spread to both. Macnicol reported a case of acute myositis and osteomyelitis due to *S milleri*, probably mixed with anaerobes, which occurred three days after dental extraction.[27]

Storage in glycerol blood broth at −20°C did not seem to affect adversely hyaluronidase production when strains were resubcultured after a few months, and the hyaluronidases of *S milleri* in supernatants were highly stable at 4°C and 37°C. Testing of Todd Hewitt broths containing neopeptone for lack of proteinase activity was a routine practice in Dr Parker's laboratory and it was not investigated whether this was critical to hyaluronidase production and recognition. Titres of hyaluronidase of *S milleri* are somewhat lower than those of *S pyogenes*.

It has been reported[28] that *S milleri* hyaluronidase includes more than one serotype, mutually distinct and distinct from the hyaluronidase of *Streptococcus pyogenes*. This work, which is continuing, may help define the more superficial sources from which *S milleri* infection reaches deep abscess sites.

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